Epratuzumab, a CD22-targeting recombinant humanized antibody with a different mode of action from rituximab

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Abstract

Epratuzumab is a humanized anti-CD22 monoclonal antibody currently in clinical trials for treatment of non-Hodgkin lymphoma (NHL) and certain autoimmune diseases. Here we report the results of investigations of epratuzumab's mode of action in comparison to and in combination with the anti-CD20 mAb, rituximab. In vitro cell growth inhibition, induction of apoptosis, and the ability of the mAbs to mediate complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) were evaluated. We also investigated the potential activity of epratuzumab in the regulation of B-cell antigen receptor (BCR) activation. Epratuzumab and rituximab displayed very distinct modes of action; epratuzumab acts as an immunomodulatory agent, while rituximab is an acutely cytotoxic therapeutic antibody. Epratuzumab has distinct effects on cell growth from rituximab. For example, rituximab + anti-human IgG Fcγ yielded marked inhibition of proliferation in human NHL cell lines, while epratuzumab had little or no effect in this assay. However, when cells were immobilized and stimulated with anti-IgM, epratuzumab, but not rituximab, caused a significant antiproliferative effect. Unlike rituximab, no CDC could be detected, and ADCC was modest but significant with epratuzumab. Importantly, combining rituximab and epratuzumab did not decrease rituximab's ability to induce apoptosis, CDC, and ADCC. In fact, the combination is more effective than rituximab alone in inhibiting proliferation of Daudi Burkitt lymphoma cells in the presence of second antibody, and at least equally effective to rituximab in the absence of crosslinking. These observations suggest that it may be possible to enhance clinical efficacy by combination therapy comprised of anti-CD20 and anti-CD22 mAbs.
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1. Introduction

The development of therapeutic monoclonal antibodies (mAbs) has become an active and fruitful area of research. MAb therapy has been found particularly effective in non-Hodgkin lymphoma (NHL), presumably because B cells express unique cell surface targets. Rituximab, a murine-human chimeric anti-CD20 mAb, was the first such agent to obtain FDA approval and widespread clinical use (McLaughlin et al., 1998; Reff et al., 1994). Based on in vitro studies, the mechanisms of

action of rituximab include complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC), as well as direct induction of apoptosis (Cartron et al., 2004). Recent investigations highlight the potential role of rituximab in chemosensitization, possibly through an impairment of intracellular signaling (Mounier et al., 2003).

Epratuzumab, a humanized IgG1 mAb targeting the cell surface antigen, CD22, has also demonstrated therapeutic efficacy against NHL in clinical trials (Leonard et al., 2003) as well as certain autoimmune diseases (Kaufmann et al., 2004; Steinfeld et al., 2005). A striking property of epratuzumab is that binding to B-lymphocytes results in rapid internalization (within minutes) of the cell surface CD22 (Carnahan et al., 2003), making epratuzumab an excellent agent for tumor-imaging (Baum et al., 1994), and for developing immunoconjugates with cyto-

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toxic drugs (Qu et al., 1998), toxins (Kreitman et al., 1993), or radionuclides (Linden et al., 2005). Epratuzumab has also been reported to induce CD22 phosphorylation, indicating a role in signal transduction (Carnahan et al., 2003). Similar to CD20, CD22 exhibits a highly restricted expression pattern, present only on B lymphocytes (Dorken et al., 1989). CD22 belongs to a class of membrane receptors that are known to modulate B-cell antigen receptor (BCR) activation. It is believed that BCR activation through antigen engagement results in a signaling cascade mediated through phosphorylation of the BCR, involving the MAP kinase pathway, as well as phosphorylation of its positive and negative regulators, CD19 and CD22, respectively. Phosphorylation of CD22 results in SHP-1 phosphatase translocation in proximity to the activated BCR, providing negative feedback (Tedder et al., 2005). The role of CD22 as a negative regulator of signaling is also suggested by the hyperactivated or chronic activation state of the B cells in CD22 genetically deleted murine models (Nitschke et al., 1997; O'Keefe et al., 1996; Otipoby et al., 1996; Sato et al., 1996).

Based on the involvement of CD22 with the BCR, its apparent role as a negative modulator, and the less dramatic B lymphocyte depletion (compared to that of rituximab) observed clinically (Leonard et al., 2003), we investigated the effects of epratuzumab in cell-based assays. Here we report the results of the investigations of epratuzumab's mode of action in comparison to and in combination with rituximab. We found that the two antibodies show very distinct modes of action, with epratuzumab appearing more as an immunomodulatory agent, in addition to its cytotoxic role, and rituximab acting as an acutely cytotoxic therapeutic antibody. We observed that combining both antibodies would not be detrimental to the cytolytic effects of rituximab.

2. Materials and methods

2.1. Cell lines

The CD22-expressing human Burkitt lymphoma cell lines, Daudi, Ramos, and Raji, were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cell lines RL and SU-DHL-6, which contain the chromosomal translocation $t(14;18)$, were obtained from Dr. John Gribben (Dana-Farber Cancer Institute, Boston, MA) and Dr. Alan Epstein (University of Southern California, Los Angeles, CA), respectively. Cell lines SU-DHL-4, SU-DHL-10, and Karpas422 were provided by Dr. Myron Czuczman (Roswell Park Cancer Institute, Buffalo, NY). The cell lines were grown in RPMI-1640 medium (Invitrogen, Carlsbad, CA) or DMEM (Life Technologies, Gaithersburg, MD), supplemented with 10% heat inactivated fetal bovine serum (FBS) (HyClone), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.2. Immunophenotyping

Indirect immunofluorescence assays were done using FITC-goat anti-human IgG, Fcγ fragment specific antibody (Tago, Inc., Burlingame, CA) essentially as described previously (Stein et al., 1989) and analyzed by flow cytometry using a FACSCaliber (Becton Dickinson, San Jose, CA).

2.3. Primary B cells from fresh human tonsils

Tonsils were collected according to standard surgical procedures, after obtaining informed consent, and were shipped overnight in RPMI-1640 medium containing penicillin and streptomycin. Cells were dissociated mechanically in cold Hanks' Balanced Salt solution (Invitrogen, Carlsbad, CA), and mononuclear cells purified using Ficoll-Hypaque™ (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's specifications. Mononuclear cells were resuspended in Hanks' medium, and washed using RPMI medium containing 10% FBS. B cells were further purified using a T-cell depletion kit (Dynal Biotech, Oslo, Norway), according to the manufacturer's directions.

2.4. In vitro cell proliferation assays

2.4.1. Effects of mAbs on ^{3}H-thymidine uptake
MAb effects on cell growth were determined by measuring ^{3}H-thymidine incorporation in the NHL cell lines with and without the presence of a crosslinking second antibody, essentially as described by Shan et al. (1998). Specificity of the effect was assessed by comparison to the isotype-matched control and the stimulatory effect of the goat anti-mouse versus goat anti-human second mAbs for humanized (or chimeric) mAbs. All tests were performed in triplicate.

2.4.2. BCR stimulation
Ninety-six well tissue culture plates (black well; Corning Inc., Corning, NY) were coated for 48 h with epratuzumab or rituximab in PBS, pH 7.4, at 4 °C at the indicated concentrations. Plates were then washed once with cold PBS, then once with RPMI, 10% FBS. The cell lines were plated at a density of 30,000 cells per well. Anti-IgM Fc$_{5μ}$ (Jackson ImmunoResearch Lab, Inc., West Grove, PA) was used at doses ranging from 0.1 to 10 μg/ml for 48 h. Cell viability was measured by addition to each well of 15 μl Alamar Blue (Biosource, Camarillo, CA), and read on a fluorescence plate reader (Spectramax Gemini, Molecular Devices, Sunnyvale CA) with an excitation of 530 nm and an emission of 590 nm. Reading was normalized by subtracting signal in wells containing tissue culture medium but without plated cells (0%).

2.5. Cytotoxicity assays

^{51}Chromium-release assays were performed for the measurement of ADCC essentially as described (Cardarelli et al., 2002). Effector cells were prepared from peripheral blood collected under a protocol approval by an institutional review board and in accordance with an assurance filed with and approved by the Department of Health and Human Services. Informed consent was obtained from each subject. CDC was measured using Alamar Blue according to Gazzano-Santoro et al. (1997).

2.6. Apoptosis quantification

2.6.1. Annexin V–FITC

Cells (2×10^5/ml) were incubated at $37\,^\circ$C with $2\,\mu$g/ml of test antibody with or without the crosslinker, protein G ($2\,\mu$g/ml), or a secondary antibody, F(ab')$_2$ fragment of a goat anti-human IgG Fcγ-specific antibody (Rockland Immuno-chemicals, Gilbertsville, PA) for up to 48 h. Cells were then washed twice with cold PBS, and incubated with Annexin V-FITC and propidium iodide (PI), according to the manufacturer's protocol (BD Biosciences, San Jose, CA), and analyzed by flow cytometry on a Becton Dickinson FACSCalibur. Untreated cells were used as control to gate Annexin V and PI negative cells. The percent of apoptotic cells was calculated by including all Annexin V-FITC positive cells.

2.7. Analysis of hypodiploid DNA by PI staining

Hypodiploid DNA was assesses after PI staining as described previously (Stein et al., 2004). Samples were analyzed by flow cytometry using a FACSCaliber. Percent apoptotic cells was defined as the percent of cells with DNA staining before G1/G0 peak (hypodiploid).

3. Results

3.1. Antigen expression of cultured human lymphoma cell lines

Flow cytometry analysis was performed using indirect immunofluorescent staining to confirm that epratuzumab and rituximab bind to a panel of cultured human B-cell lymphomas. As shown in Table 1, these mAbs bind to the tested cell lines, but the level of fluorescence staining varied between the cell lines. In all lines tested, epratuzumab staining was much weaker than that observed with rituximab.

Because the rapid internalization of epratuzumab into target cells compromises the ability of the indirect assay to quantitate relative antigen expression, staining was also performed using directly labeled FITC-anti-CD20 (B1) and FITC-anti-CD22 (RFB4). CD22 expression is similar to or greater than CD20 expression in seven of the ten cell lines tested (Table 1). Three of the non-Burkitt cell lines (SU-DHL-6, RL, and DoHH2) express CD20 at much greater levels than the other seven cell lines. In these three lines, CD20 expression is substantially greater than CD22.

3.2. Effects of mAbs on proliferation of NHL cell lines

We utilized various approaches that present the mAbs to target cells in different formats, either in suspension or immobilized on polystyrene plates, to evaluate growth inhibition by epratuzumab and rituximab. In addition, the antiproliferative effects of the mAbs in suspension were examined with a second antibody for crosslinking, to mimic the role of receptor ligation in vivo, while that of the immobilized mAbs were evaluated with the cells stimulated by an anti-IgM antibody for crosslinking of cell surface IgM, as a means of activating B cells in lieu of BCR stimulation by antigen engagement.

As measured by the uptake of ^{3}H-thymidine, rituximab caused specific growth inhibition of the NHL cells, which was enhanced when the mAb was crosslinked, but the level of inhibition varied between cell lines with no apparent correlation with cell surface CD20 expression. SU-DHL-6 was markedly more sensitive to rituximab than other cell lines. In the absence of crosslinking, rituximab yielded approximately 88% inhibition of proliferation of SU-DHL-6 cells, which increased with crosslinking to 98% (data not shown). In Ramos and Daudi cells, greater than 60% inhibition was seen with crosslinked rituximab compared to less than 40% without crosslinking.

In contrast, the anti-CD22 mAb epratuzumab gave little or no inhibition of proliferation of the cell lines examined in this assay, and crosslinking with the anti-human IgG second antibody did not increase this effect. However, as shown in Fig. 1, the combination of rituximab and epratuzumab in the presence of crosslinking by second antibody was significantly more effective

Table 1
Antigen expression: flow cytometry assay (geometric mean fluorescence)

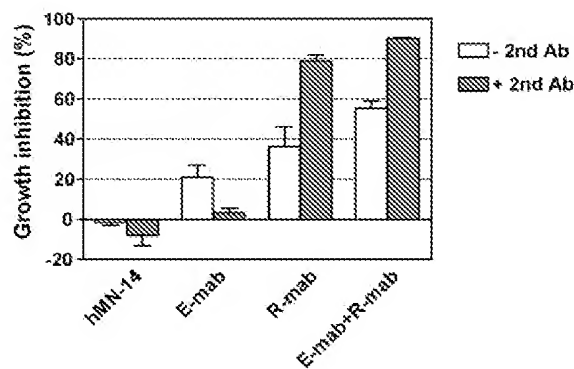| Cell line	Indirect staining			Direct staining		
	Control (hMN-14)	Rituximab	Epratuzumab	CD8	CD20	CD22
Burkitt						
Daudi	5.9	252.9	25.5	3.2	39.4	171.8
Raji	2.2	384.7	16.2	3.6	53.1	84.0
Ramos	1.1	119.5	5.0	3.1	41.5	67.7
Non-Burkitt						
DoHH2	4.8	45.6	4.8	4.3	328.2	61.0
Karpas422	8.3	12.2	8.2	3.6	14.0	15.3
Namalwa	2.5	7.3	6.8	n.d.[a]	n.d.	n.d.
RL	3.1	158.9	5.3	3.5	196.0	21.3
SU-DHL-4	2.5	46.8	5.9	3.1	10.9	26.9
SU-DHL-6	1.6	599.5	5.5	2.3	419.9	38.9
SU-DHL-10	2.5	35.7	6.7	3.4	10.3	19.7
WSU-FSCCL	3.0	36.4	6.0	3.0	5.1	8.0

[a] not determined.

Fig. 1. Anti-proliferative effects of the combination of epratuzumab (E-mab) and rituximab (R-mab) by *in vitro* ^{3}H-thymidine uptake assay. Daudi cells were cultured with the mAbs (5 µg/ml) with or without a second antibody for crosslinking to mimic the role of effector cells *in vivo*. White bars, without second antibody; gray bars, with 20 µg/ml of F(ab')$_2$ fragment of goat anti-human IgG (Fc) antibody. Error bars represent standard deviations of triplicates.

than crosslinked rituximab alone in inhibiting proliferation of Daudi cells ($90.3 \pm 0.4\%$ versus $78.9 \pm 3.0\%$, $P = 0.021$). Since the difference in anti-proliferative effect caused by the combination of epratuzumab and rituximab in the absence of crosslinking ($55.6 \pm 3.2\%$) compared to rituximab alone ($36.3 \pm 9.9\%$) did not reach statistical significance ($P = 0.065$), we conservatively conclude that under these conditions there is a trend suggesting that the combination of epratuzumab and rituximab is more effective than either alone.

3.3. Effect of epratuzumab and rituximab on apoptosis

The ability of epratuzumab and rituximab to induce apoptosis in NHL cells was measured in the presence of protein G for crosslinking using Annexin V/PI staining, followed by flow cytometry analysis. Fig. 2A and B, shows results of a representative experiment in Ramos, a sensitive cell line to rituximab-induced apoptosis. Rituximab incubation for 18 h in the presence of protein G resulted in 50% apoptosis, whereas no measurable apoptosis was induced by epratuzumab under these conditions. Combining epratuzumab with crosslinked rituximab does not significantly alter the levels of apoptosis obtained with crosslinked rituximab alone (Fig. 2C).

Induction of apoptosis was also evaluated by detection of hypodiploid DNA. Cells were cultured with the mAbs with or without second antibody, followed by DNA staining with PI. Cells were analyzed by flow cytometry, and positive fluorescence below the G1 region represents DNA fragmentation and is a measure of apoptosis. In all cell lines except SU-DHL-6, neither rituximab nor epratuzumab induced apoptosis in the absence of crosslinking (Table 2). In SU-DHL-6, the anti-CD20 mAb rituximab also induced apoptosis without crosslinking. Specific induction of apoptosis was seen with both rituximab and epratuzumab when an anti-IgG Fcγ crosslinking agent was used. The percent of hypodiploid nuclei induced by the two mAbs were similar, but relatively low (12–15%), in Daudi, RL, and Raji. SU-DHL-6 and Ramos cells were more sensitive to crosslinked-rituximab than the other cell lines (25.7%
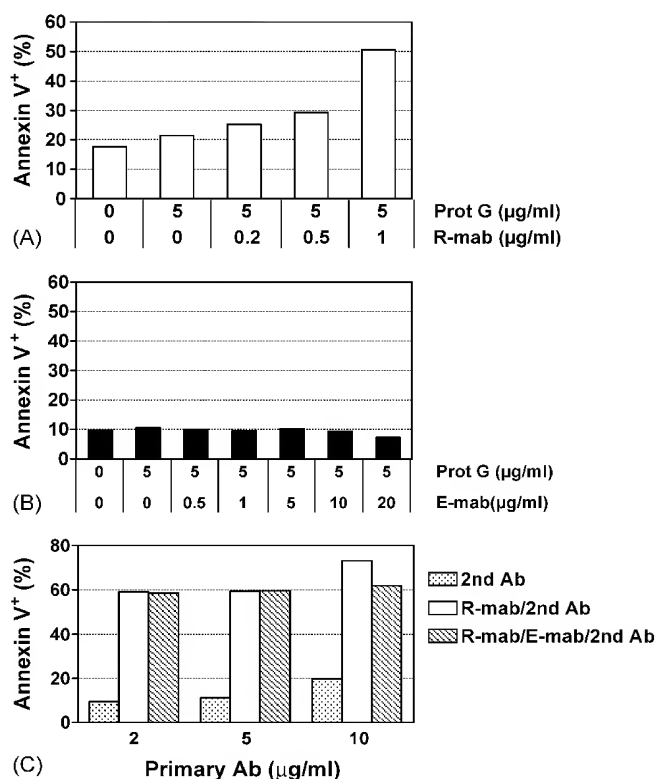

Fig. 2. Apoptotic effect of mAbs on Ramos determined by Annexin V. Ramos cells were plated at 0.5×10^6 cells/ml in medium containing varying doses of rituximab (R-mab) (A), epratuzumab (E-mab) (B), or both mAbs (C) and 5 µg/ml of the IgG crosslinking agent, protein G. Cultures were incubated for 24 h. Apoptosis was quantified by measuring percent of Annexin V-positive cells.

and 33.5% hypodiploid DNA, respectively), and both cell lines were also more sensitive to rituximab than to epratuzumab. It is interesting to note that Ramos exhibited the largest difference in sensitivity between rituximab and epratuzumab, with rituximab yielding almost two-fold the level of hypodiploid DNA as epratuzumab. Although technical differences between the Annexin V/PI staining and hypodiploid DNA measurement assays enabled detection of low levels of apoptotic induction by epratuzumab in Ramos only by the latter, the results of both

Table 2
Apoptotic effect of anti-CD20 MAbs as shown by PI staining (% hypodiploid DNA)

Cell line	2nd MAb	1st MAb			
		None	hMN-14	Rituximab	Epratuzumab
SU-DHL-6	None	4.2	3.9	9.4	4.4
	Anti-hIgG Fc	7.0	6.7	25.7	19.4
Daudi	None	1.4	1.3	1.2	1.1
	Anti-hIgG Fc	1.3	2.2	15.0	14.0
RL	None	2.4	1.8	3.7	1.8
	Anti-hIgG Fc	2.0	4.6	14.1	13.5
Ramos	None	4.1	2.3	2.5	1.7
	Anti-hIgG Fc	1.8	6.6	33.5	17.4
Raji	None	1.5	1.9	2.3	2.1
	Anti-hIgG Fc	2.0	5.3	12.1	12.8

studies are consistent in the demonstration that Ramos cells are more sensitive to rituximab than to epratuzumab. In contrast, detection of hypodiploid DNA after incubation with crosslinked-epratuzumab and crosslinked-rituximab were within 1% of each other in Daudi, RL, and Raji.

3.4. Amplification of BCR-induced cell death

An interesting and paradoxical effect of stimulating BCR is that it can result in proliferation of primary tonsillar B cells (Doody et al., 1995), but induces apoptosis when B-tumor cells are targeted (Chaouchi et al., 1995). It has also been reported that blocking CD22 translocation by immobilization of anti-CD22 antibody results in BCR-amplified activation of primary B cells (Doody et al., 1995). Epratuzumab was tested using a similar paradigm to determine its effect on primary tonsillar B cells and B-tumor cells when used in concert with stimulation of BCR. Culture plates were used as the solid support. Although epratuzumab can still bind cell surface CD22, its adsorption onto a plate should prevent CD22 internalization and restrict lateral diffusion of CD22 in the plane of the membrane.

Incubation of NHL cells with immobilized epratuzumab and rituximab in the presence of anti-IgM (in solution) yielded contrasting results to those obtained when the cells were treated with soluble mAbs. Fig. 3A shows the results obtained using Ramos cells as the target. Whereas crosslinked rituximab had a stronger inhibitory effect on cell growth than crosslinked epratuzumab

when the cells and antibodies were studied in suspension, immobilized rituximab had no effect. Thus, rituximab treatment of Ramos cells did not yield a significant change in the rate of proliferation compared to the cells incubated in control IgG1-coated wells (not shown). Conversely, epratuzumab yielded a dose-dependent enhancement of the anti-proliferative effect in Ramos; approximately 20% in 1 µg/ml epratuzumab-coated wells ($P = 0.005$) and a greater than 50% increase in inhibition at 5 µg/ml and higher ($P < 0.0005$). This effect did not require the Fc portion of epratuzumab since $F(ab')_2$ fragment-coated plates yielded similar anti-IgM amplified cell death (not shown), indicating that the effect is mediated through epratuzumab binding to CD22 on the target cells. Since it is possible that the solid phase may mimic binding of epratuzumab to Fc-receptors of effector cells *in vivo*, we have not ruled out the possibility that the Fc fragment may be required for epratuzumab activity on cells, for example, within lymph nodes or lymphoma deposits. Consistent with the results described above with the ^{3}H-thymidine uptake assay, no effect on cell death above that observed with anti-IgM alone was seen when up to 50 µg/ml of epratuzumab was added in solution, even in presence of a crosslinking agent (data not shown).

The enhanced cell growth inhibition by epratuzumab was not as pronounced in all B-cell lines tested and there was no apparent correlation with cell surface CD22 expression (Fig. 3B). Although Daudi has the highest CD22 receptor density, it was insensitive to cell death amplification. The same was true for the
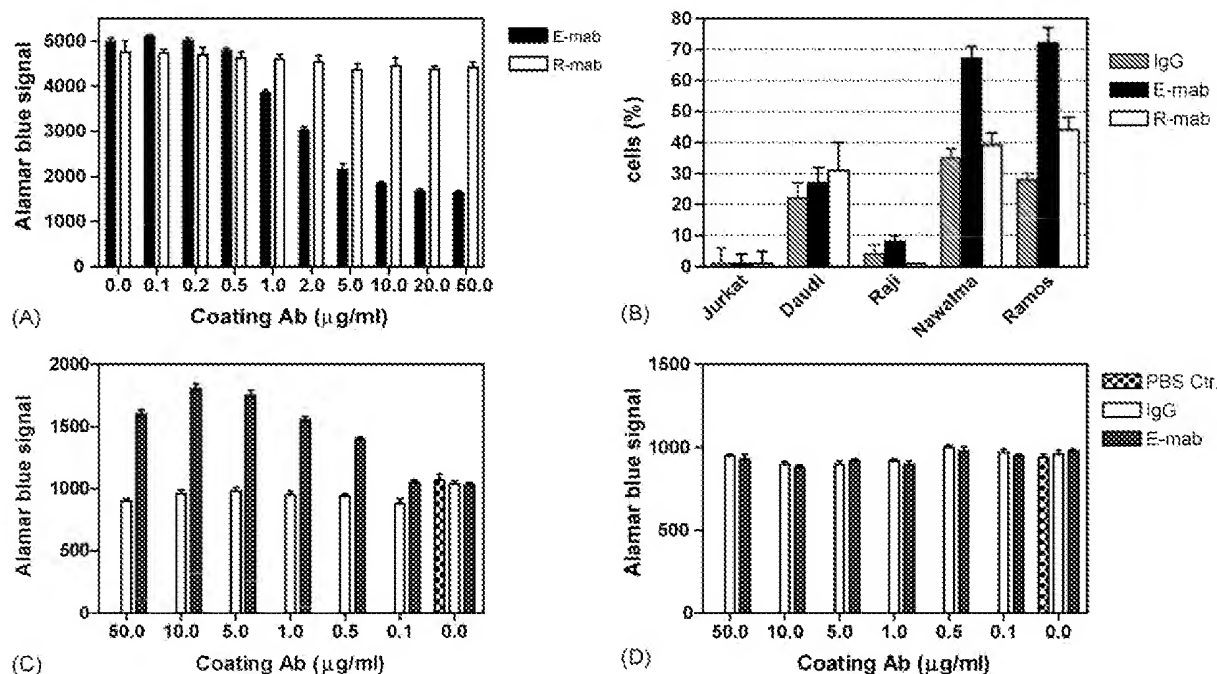

Fig. 3. Immobilized epratuzumab but not rituximab amplifies BCR stimulation-induced effects. NHL cell lines or fresh tonsillar B cells were plated on antibody-coated 96-well plates and stimulated with an anti-IgM crosslinking antibody. Viable cell population was measured after 48 h using Alamar Blue. (A) Comparison of the effect of immobilized epratuzumab and rituximab, on Ramos cells. Cells were stimulated with 5 µg/ml anti-IgM antibody. (B) Anti-IgM-mediated death in relation to CD22 density. B-lymphoma cell lines were plated on antibody-coated tissue culture plate (10 µg/ml), and stimulated with an anti-IgM antibody, 5 µg/ml. Jurkat cells (CD22- and CD20-negative cell line) were used as a negative control. Percent cell death is calculated using the cells not treated with anti-IgM as 100% viable cells, and well containing no cells as 100% cell death. (C) Fresh tonsils were dissociated and isolated B cells plated on immobilized epratuzumab or IgG1 control antibody in the presence of 5 µg/ml anti-IgM antibody. (D) Fresh tonsil B cells were incubated with epratuzumab or IgG1 control antibody in solution in the presence of 5 µg/ml anti-IgM. Viable cell population was measured after 48 h using an Alamar Blue assay.

Raji cell line, which has a CD22 receptor density comparable to that of Ramos cells. Both Namalwa and Ramos cells showed the same sensitivity to being plated on epratuzumab-coated plates. It is possible that cell surface IgM density may be related to this BCR-activation amplification, since we found IgM expression to be 3.4-fold higher in Ramos than Daudi cells (data not shown).

Fig. 3C and D, shows the effects of epratuzumab on primary human tonsillar B cells. Primary B cells plated on immobilized epratuzumab have enhanced proliferation in response to anti-IgM stimulation, compared to cells plated on a control human IgG1 mAb (Fig. 3C) or rituximab (not shown). The epratuzumab effect shows a dose-dependency, saturating at a coating concentration of approximately 5 μg/ml. However, when epratuzumab was added in solution no amplification was observed at any of the concentrations tested (Fig. 3D). These results suggest that preventing CD22 translocation (i.e., internalization or lateral movement), rather than down-regulation of the quantity of CD22 on the cell surface, mediates this effect.

3.5. Inhibition of cell proliferation by immobilized epratuzumab independent of BCR activation

The effects of mAbs on inhibiting cell proliferation and inducing apoptosis are dependent on a number of factors including culture conditions. This is due to competition of the added anti-B-cell mAbs with the components in the culture medium for promoting cell growth and protection from apoptosis. The antiproliferative effects of immobilized epratuzumab and rituximab were evaluated on cells stressed by lowering the concentration of FBS in the culture medium from 10% to 3%. Ramos and D1-1, a subclone of Daudi, were used. As shown in Fig. 4, both Ramos and D1-1 cell proliferation were affected by immobilized epratuzumab in the absence of added anti-IgM. Three days of culturing in epratuzumab-coated wells resulted in ~30% ($P = 0.003$) and >50% ($P = 0.001$) inhibition of proliferation of these cell lines. Under the same conditions, immobilized rituximab and isotype-matched control IgG1 mAbs, hLL1 (B-

Fig. 4. Inhibition of cell growth by immobilized mAbs. Ramos and D1-1 cells were cultured in 48-well cell culture plates pre-coated with 5 μg/ml mAb, as indicated. Medium containing ~3% FBS was used in these experiments. After 3 days of incubation in a CO_2 incubator, 37 °C, the viable cell numbers were determined by MTT assay. The results shown are the average of three sets of experiments.

cell binding, anti-CD74) and hMN14 (non-B-cell binding), did not have significant effects. Statistically significant differences between epratuzumab and rituximab were observed ($P = 0.009$ for both cell lines).

3.6. Effect of epratuzumab and rituximab on CDC and ADCC

CDC was measured on three B-cell lines, Ramos, Daudi, and Raji. Rituximab was shown to cause lysis in all three cell lines, while epratuzumab had essentially no effect. Daudi was the most sensitive of the B-cell lines tested, with 100% lysis at 1 μg/ml rituximab (Fig. 5A), while Raji and Ramos cells required 10 μg/ml rituximab to achieve the same effect (data not shown).

Daudi was used to examine whether epratuzumab competes with rituximab for recruiting complement and if it affects rituximab's cytolytic activity when the two mAbs are combined. Jurkat T-cell lymphoma was used as a negative control. When increasing concentrations of epratuzumab were added along with 1 μg/ml rituximab, no reduction in CDC was observed (Fig. 5B). This suggests that although epratuzumab does not mediate complement-dependent cell lysis, it would not interfere with rituximab's cytolytic activity when these two mAbs are combined in an immunotherapy setting. In other experiments, we pre-treated B cells with saturating amounts of epratuzumab for 1 h, overnight, or 65 h prior to adding rituximab and complement. This pre-incubation step did not modulate rituximab's CDC activity (data not shown).

The ability of epratuzumab to induce ADCC was evaluated, comparing it to and combining it with rituximab, using Raji as the target cells. Rituximab induced up to 40% ADCC in the presence of effector cells from donor 1400 (Fig. 5C). Epratuzumab showed more moderate, but significant activity ($P < 0.0002$ at E:T ratios 1:50 and 1:25, $P = 0.004$ at E:T ratio 1:100, compared to IgG1 control) in the same experiment. The ADCC assay was repeated with seven different donors to allow screening through allelic polymorphism existing in the FcγRIII population with similar findings (Fig. 5D). Similar results were also observed when ADCC was evaluated in Daudi and SU-DHL-6 (Fig. 5E and F). The combination of the two antibodies did not result in significantly increased efficacy. Thus, rituximab was consistently more potent than epratuzumab in mediating ADCC. However, adding epratuzumab to rituximab had no detrimental effect on rituximab-mediated ADCC.

4. Discussion

CD22, a B-cell restricted transmembrane sialo-glycoprotein, has recently been shown to play a complex role in the regulation of normal B-cell function, both as an adhesion molecule and as a component of the B-cell activation complex (Nitschke, 2005; Tedder et al., 2005). Its role as a lectin-like adhesion receptor is facilitated through CD22 binding of α2,6-linked sialic acid-bearing ligands. Two extracellular (N-terminal) domains have been shown to be required for ligand binding (Engel et al., 1995). The role of CD22 in modulating signaling through the BCR complex is due to phosphorylation of three immunoreceptor
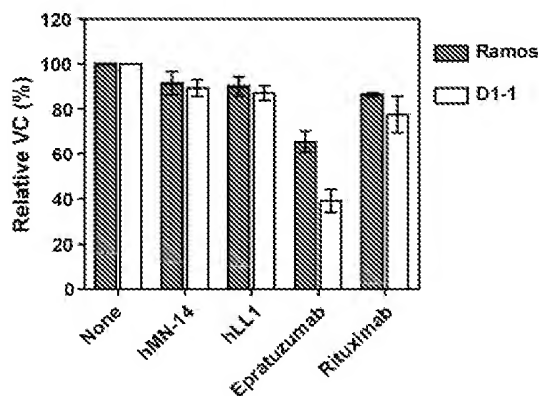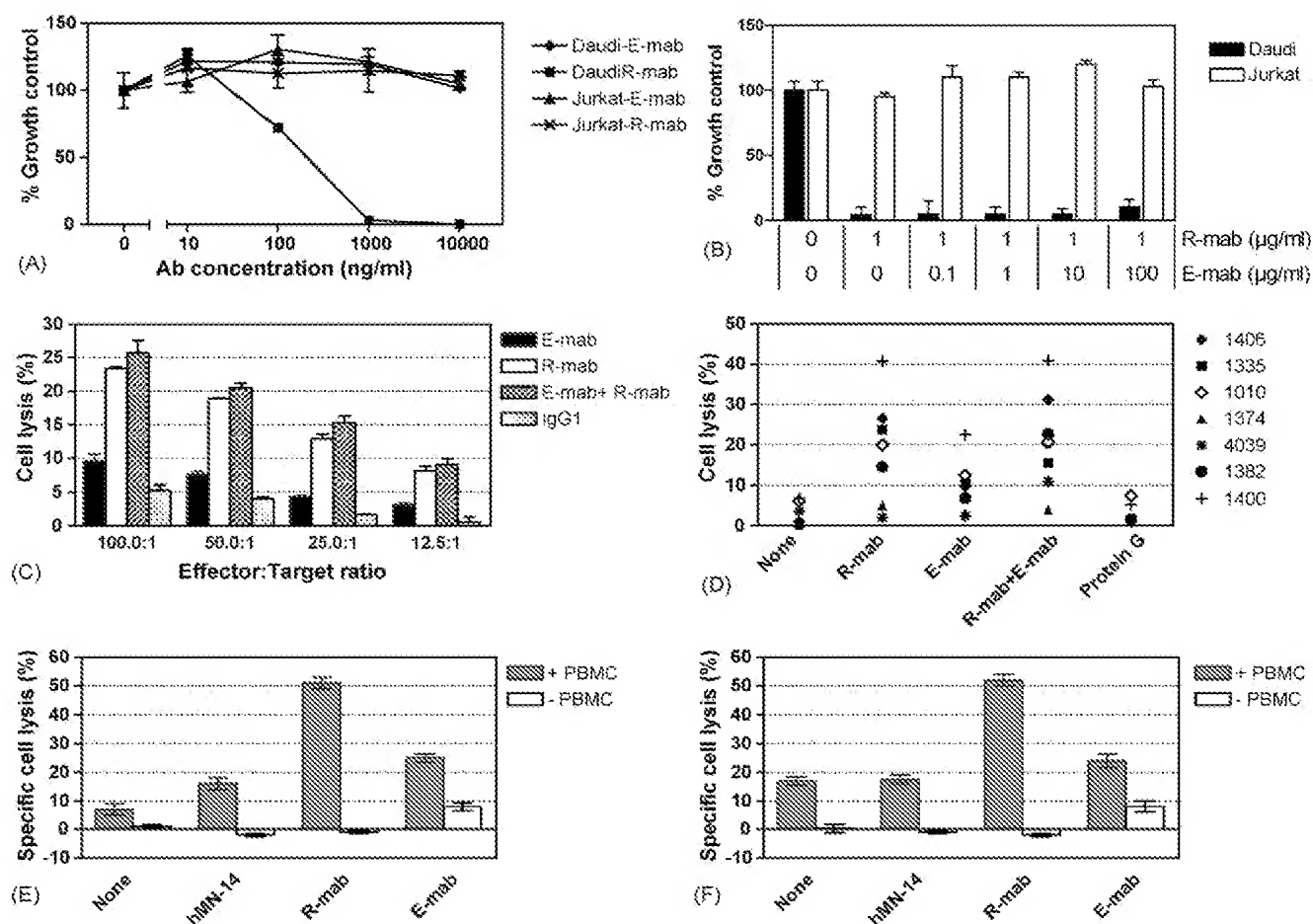
Fig. 5. Effect of rituximab and epratuzumab on CDC and ADCC. (A) Daudi cells or control cells (Jurkat) were treated with either rituximab (R-mab) or epratuzumab (E-mab) at the concentrations shown in the presence of complement. Cell viability was measured using Alamar Blue and reported as % of viable population relative to cells treated with complement only (no mAb). (B) Daudi or control cells (Jurkat) were treated with a fixed dose of rituximab (1 μg/ml) together with increasing concentrations of epratuzumab. The lytic effect of rituximab on Daudi was approximately 100% and unchanged through the tested conditions. ^{51}Cr-labeled NHL cells were incubated with anti-B-cell mAbs in the presence of human peripheral blood mononuclear cells for measurement of ADCC induction by epratuzumab and rituximab. (C) ADCC in Raji cells was assessed across a range of effector cell to target cell ratios using epratuzumab and rituximab singly and in combination. (D) Summary of ADCC activity for epratuzumab, rituximab, or both using effector cells from a panel of healthy donors. (E) ADCC in Daudi cells. (F) ADCC in SU-DHL-6 cells.

tyrosine-based inhibitory motifs (ITIM) on its intracellular tail upon BCR stimulation. Phosphorylated CD22 can then recruit tyrosine phosphatase SHP-1 and other effector molecules, which in turn limit BCR signaling (Nitschke, 2005; Tedder et al., 2005). Thus, a major role of CD22 is as a negative regulatory molecule limiting the intensity of BCR-generated signals. The functional relationship between the ligand-binding domains and the signaling domains has been an area of active investigation. Hypotheses have included direct mediation of CD22-BCR interactions by sialic acid binding (Cyster and Goodnow, 1997; Kelm et al., 2002), and the ability of α2,6-linked sialic acid carrying proteins to sequester CD22 away from the BCR (Doody et al., 1995).

In this report, we engaged several different cell-based assays to evaluate the effects of the anti-CD22 mAb epratuzumab on B-lymphoma cells to gain an understanding of these effects in light of the biology of CD22. Moreover, these assays explored whether a rationale exists for combining epratuzumab with rituximab clinically. A number of anti-CD22 mAbs that bind to

different CD22 epitopes have been reported. Many of these bind to the two N-terminal Ig-like domains essential for CD22-mediated adhesion, and fully or partially block CD22-mediated adhesion. MAbs in this group include the HB22 series, as well as others (Engel et al., 1993). Epratuzumab and the anti-CD22 mAb, RFB4, bind to the third domain (epitope B) (Stein et al., 1993). Because CD22 has been reported to regulate B-cell function through both ligand-dependent and ligand-independent mechanisms, it is likely that treatment with these various mAbs may result in a range of clinical outcomes.

The activity of epratuzumab was first evaluated as a soluble agent either alone or in a crosslinked form. Crosslinking was performed with anti-IgG or protein G, agents which can be considered to mimic the receptor ligation that may occur in the natural setting. In solution, epratuzumab was not found to be an effective cytotoxic agent, either alone or crosslinked. This contrasts with the effectiveness of crosslinked rituximab. The efficacy of crosslinking rituximab has been noted previously, and extensively evaluated (Mathas et al., 2000; Shan et al.,

1998; Zhang et al., 2005). Our results are consistent with these earlier observations. In all B-cell lines studied, we observed specific inhibition with crosslinked rituximab, with significantly lower anti-proliferative effects in the absence of a crosslinking agent. It has been hypothesized that a substantial proportion of the cytotoxic effects of anti-CD20 mAbs observed in clinical trials may be due to an analogous mechanism; namely, *in vivo* ligation of malignant B cells by anti-CD20 mAbs followed by FcR-mediated crosslinking by macrophages or other accessory molecules (Gong et al., 2005; Shan et al., 1998). Hyper-crosslinking of rituximab redistributes CD20 into lipid rafts followed by activation of Src-family tyrosine kinases such as *Lyn*, *Fyn*, and *Lyc* (Deans et al., 1995) kinases, which are also involved in the modulation of signaling events after BCR activation (Kurosaki, 1998). Our observation that the combination of rituximab and epratuzumab was significantly more effective than rituximab alone in inhibiting proliferation of Daudi cells (in the presence of crosslinking by second antibody) may therefore be due to the simultaneous activation of these converging mechanisms.

In contrast to the lack of *in vitro* activity of soluble epratuzumab, we found that immobilized epratuzumab was able to yield a significant reduction in viable cell count in two NHL cell lines, Ramos and Namalwa, incubated with anti-IgM, as well as a significant increase in the proliferation of tonsillar B cells. Immobilization on plastic was used to prevent the antibody and its target antigen from being internalized after they interact on the cell surface, and to restrict lateral diffusion of antigens in the plane of the membrane. Doody et al. (1995) suggested that incubation of CD22+ cells with immobilized anti-CD22 sequesters CD22 from surface IgM, thereby disabling the inhibitory feedback effect CD22 would otherwise exert on the activation of the B cell via its interaction with BCR. Therefore, restricting lateral diffusion of CD22 in the plane of the membrane (by incubating with an immobilized anti-CD22 mAb) enhances the response of B cells to the ligation of surface IgM. The physiologically relevant analog of immobilized antibody to CD22 may be the B and T cells surrounding antigen-stimulated B cells in secondary lymphoid organs. Immobilized epratuzumab was also cytotoxic to NHL cell lines without anti-IgM when the cells were stressed by culturing in low serum concentration. Thus, a second mechanism must be involved. It is possible that disturbing the cellular distribution of CD22 on the B-cell membrane or preventing internalization of the CD22-epratuzumab complex may allow any signaling caused by direct interaction between the antibody and antigen (not necessarily involving the BCR) to be potentiated. Interactions with other ligands for CD22 may be involved, possibly CD45, a known ligand of CD22 (Sgroi et al., 1995).

The effects of anti-CD22 mAbs on B-cell function have been studied by multiple groups with apparently conflicting results. CD22 ligation was reported to generate stimulatory signals in some assay systems (Doody et al., 1995; Pezzutto et al., 1987, 1988) and apoptotic signals in others (Chaouchi et al., 1995; Tuscano et al., 1999). In our assay system, immobilized epratuzumab plus anti-IgM had differing effects dependent on the cell type. The function of CD22 in the regulation of B-cell survival and signal transduction is clearly complex, and thus it is not surprising that varying responses have been observed experimentally when different assay systems and different cell types, varying in levels of maturation and activation, are used. Indeed, antigen receptor signaling is known to have different consequences depending on the stage of B-cell maturation and the strength and duration of the signal, variously triggering either cell proliferation, anergy, or death by apoptosis (Chaouchi et al., 1995; Cyster and Goodnow, 1997).

Our observation that rituximab induces CDC and ADCC in B-lymphoma cell lines is consistent with previous reports on rituximab's induction of CDC (Flieger et al., 2000; Reff et al., 2002) and ADCC (Flieger et al., 2000; Reff et al., 1994). However, unlike rituximab, no complement-mediated lysis could be detected following incubation with epratuzumab. Moderate, but significant, ADCC activity was observed when NHL cells were treated with epratuzumab, supporting the findings reported previously by Gada et al. (2002). It is interesting to note that the humanized anti-CD20 mAb, hA20 (IMMU-106), was constructed using the same human V framework and $\gamma 1/\kappa$ constant region sequences as epratuzumab, yet hA20 induces CDC at similar levels as rituximab (Stein et al., 2004). We therefore conclude that differences in antigen specificity rather than a deficiency in physical sequences or the tertiary structure of epratuzumab limit its activity in these assays. It is possible that the lack of epratuzumab-induced CDC may be due in part to the distance between the epitope to which epratuzumab binds and the plasma membrane, resulting in lack of activation of the complement cascade. Alternatively, the lack of CDC activity and modest ADCC activity of epratuzumab may be due to its rapid internalization following antigen binding, resulting in an apparently reduced cell surface expression of CD22 (Carnahan et al., 2003). In the case of rituximab, induction of CDC has been demonstrated to correlate with the level of CD20 expression in B lymphomas (Manches et al., 2003) and normal circulating B cells (Vugmeyster et al., 2003). The ability of rituximab and other anti-CD20 mAbs to induce CDC also correlates with their ability to translocate CD20 into lipid rafts (Cragg et al., 2003). The relationship between CD22, anti-CD22 mAbs, and lipid rafts remains to be established, but may also factor into the inability of epratuzumab to induce CDC. Indeed, it has been reported that CD22 is excluded from lipid rafts (Pierce, 2002). Although the ability of rituximab to induce CDC has been credited with a large part of its clinical efficacy, it has also been correlated with the occurrence of severe first-dose side effects of rituximab treatment (van der Kolk et al., 2001). Since combining rituximab and epratuzumab *in vitro* did not decrease rituximab's induction of CDC or ADCC, the combined use of these two mAbs may yield increased therapeutic benefit without adding to this toxicity. Indeed, the full-dose combination of epratuzumab with rituximab was well tolerated and had significant clinical activity in a recent study of 23 patients with recurrent B-cell lymphoma (Leonard et al., 2005). In addition, potentiation of anti-CD20 mAb activity has been observed in *in vivo* animal studies when epratuzumab was combined with rituximab or hA20 (Hernandez-Ilizaliturri et al., 2002; Stein et al., 2004).

In summary, comparative studies using several different cell-based assays demonstrated that epratuzumab has distinct effects on cell growth from rituximab. These effects are consistent with the biology of its target, CD22, an immune regulatory receptor. In the soluble form, rituximab is more potent in inhibiting proliferation when crosslinked, while epratuzumab is not effective as a soluble agent in either a crosslinked or non-crosslinked form. However, epratuzumab, but not rituximab, is able to inhibit cell proliferation when immobilized. Unlike rituximab, no complement lysis could be detected, and ADCC levels were modest when NHL cells were treated with epratuzumab. Combining rituximab and epratuzumab *in vitro* did not decrease rituximab's acute and potent effects in inducing apoptosis, CDC, and ADCC. In fact, *in vitro* studies on the combination of rituximab and epratuzumab indicated that the combination may be more effective than rituximab alone in inhibiting proliferation of Daudi cells. Costimulatory effects of anti-CD20 and -CD22 have been reported previously; Pezzutto et al. (1987) noted that when the anti-CD22 mAb, HD6, and the anti-CD20 mAb, 1F5, were combined, a higher level of proliferation was induced in resting B cells than with either mAb alone.

The murine-human chimeric antibody, rituximab, has shown marked success in the treatment of NHL. However, there is still a substantial percentage of patients who do not respond (~50%) (Davis et al., 2000). Thus, there is an ongoing effort to improve these results. We examined the similarities and differences of epratuzumab and rituximab to better understand the manner in which they exert their cytotoxic effects, and whether they may be effectively combined for therapeutic benefit. We conclude that as a single and naked antibody, epratuzumab's mode of action results mostly from its ability to enhance the response of tumor cells to antigen activation of BCR, a function consistent with the ability of epratuzumab to induce CD22 phosphorylation. This activity would presumably occur mostly in lymph nodes and other compartments where cell density and geometry allows for epratuzumab binding both through its Fc portion and to its target, CD22. We postulate that dendritic cells or macrophages bearing the FcγRIII receptor may ligate or cluster CD22 through epratuzumab on targeted tumor cells or on a subpopulation of B cells implicated in autoimmunity. Upon antigenic stimulation, these cells may be more susceptible to programmed cell death compared to non-antibody-treated cells in this compartment. In circulation, as we have shown previously, it is likely epratuzumab induces a rapid and prolonged internalization of CD22, perhaps also impairing the homing of the tumor cells to bone marrow (Nitschke et al., 1999). Clinically, epratuzumab has induced remission of disease in NHL (Leonard et al., 2003), and in systemic lupus (Kaufmann et al., 2004) and Sjögren's syndrome (Steinfeld et al., 2005), as well as a depletion of circulating B cells, but not as severe as reported for rituximab (Reff et al., 1994).

Our *in vitro* studies showed rituximab to be a compatible co-treatment with epratuzumab, because its mode of action is very distinct and unaffected by combination with epratuzumab. These observations suggest the possibility that in patients epratuzumab and rituximab may have non-overlapping, complementary, and beneficial efficacy, thus warranting assessment of this hypothesis in clinical trials. Indeed, initial single- and multiple-center trials of this combination of antibodies in indolent and aggressive NHL have suggested a therapeutic advantage for the combination without increased host toxicity (Leonard et al., 2005; Strauss et al., 2005).

Acknowledgments

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